MHC CONTROL OF MERCURY-INDUCED AUTOIMMUNITY IN MICE

By

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To Kim, my beloved spouse and greatest supporter. Without her encouragement and understanding, I would have never completed this work.

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One of the most striking features of exposure to low doses of mercury in mice is the high-titer haplotype-linked anti-nucleolar (ANoA) autoantibody response. Mice of the H-2^s haplotype have been high responders while H-2^b mice have been low. This pattern has been attributed to the class II molecule itself, but the poor response of F_1 crosses between high and low responders raised the possibility that the anti-fibrillarin specificity was actually due to a closely linked dominant negative gene. To test the role of class II explicitly, F_1 crosses between congenic B6.SJL (H-2^s) and C57BL/6 (H-2^b) mice with a targeted deletion of I-A_β^b were generated, creating mice heterozygous for all MHC loci, but expressing only I-A^s. In comparison with B6.SJL mice, no diminution of titers was found, proving that I-A^s itself was responsible for susceptibility and I-A^b for downregulation. Unlike I-A, expression of the I-E class II molecule could not

downregulate the response in an otherwise susceptible mouse. Using adoptive transfer techniques we have examined several mechanisms by which the resistant haplotype could be downregulating the anti-fibrillarin response in F_1 (s/b) mice. Similar to other autoimmune models, mercury induced autoimmunity requires cognate MHC-restricted T cell help. The absence of autoantibody production in F_1 mice was not due to either a difference in thymic education nor to the absence of anti-fibrillarin-specific T cell help. These results suggest a complicated role for class II in the regulation of a novel, environmentally-induced autoimmune response.

CHAPTER 1
BACKGROUND AND SIGNIFICANCE

Introduction

Autoimmune disease occurs when the body mounts an immune response against self. This immune response can involve either humoral and/or cellular immune pathways. Currently, the cause of autoimmunity is not known but both environmental as well as genetic factors appear to be important. The prevalence of autoimmune diseases has been estimated to be approximately 3% (1).

Autoimmune diseases can be classified as systemic or organ-specific. Examples of organ-specific autoimmune diseases include: type I diabetes, myasthenia gravis, Graves disease and multiple sclerosis. The systemic autoimmune diseases are represented by rheumatoid arthritis, scleroderma and systemic lupus erythematosus (SLE).

SLE is a multisystem disorder characterized by the presence of a variety of autoantibodies that are responsible for many of its clinical manifestations. The prevalence rate, based on the total population, is one in 2000, but one in 700 for women between the ages of 20 and 64 years and one in 245 for black women in the same age group (2). The etiology of SLE is unknown but many factors have been identified that influence SLE. Genetic susceptibility is strongly suggested because of the high concordance rate between monozygotic twins. Certain MHC haplotypes are more frequently associated with the

disease as well. The fact that women are much more susceptible to SLE points to hormonal factors playing a role. Finally, several environmental factors have been associated with SLE flare ups.

Experimental animal models have been widely used to study autoimmune diseases. Some of the models develop spontaneous autoimmunity like Type I diabetes in NOD mice and BB rats. Spontaneous disease similar to SLE develops in (NZB x NZW)F_1 mice. Induced models of autoimmune disease require manipulation of the animal which in turn causes an immune response to self antigens such as antigen-induced orchitis and uveoretinitis.

A second type of induced autoimmune disease can be elicited in animals without actually injecting either the autoantigen itself or a cross reactive antigen. For example, exposure to Hg^{2+}, an environmental toxin, in certain strains of rodents results in autoimmune disease. Interestingly, humans also have been reported to develop membranous glomerulonephritis upon exposure to mercury in an industrial setting (3). Some of the characteristics of this disease are remarkably similar to human SLE.

$HgCl_2$-induced autoimmunity was first reported in mice by Robinson *et al.* in 1984 (4). Within one week after the start of treatment with $HgCl_2$, outbred Swiss ICR mice produced antinuclear antibodies (ANA) of the IgG subclass. Further analysis of the ANAs revealed a nucleolar pattern (antinucleolar antibodies; ANoA). Inbred A/J mice, on the other hand, failed to respond to $HgCl_2$.

Since this initial discovery, the link between susceptibility and genetics has been investigated by several groups. Initially the link was established between ANoA induction

and the mouse major histocompatibility complex (MHC) (5-7). Testing of numerous MHC-congenic strains led to the following ranking of susceptibility based on haplotype: d < k < b < r < m < f < q < s (6). Further studies involving intra-H-2 recombinant haplotypes revealed that the I region of the MHC, specifically the I-A region, controlled the response (5,6). The other I region locus, I-E, has been shown to either decrease the response or have no effect on the ability of $HgCl_2$ to induce ANoAs (6-8).

The response was shown to be co-dominantly inherited by intercrossing susceptible and resistant strains of mice (9). For example, crosses between susceptible SJL and resistant B6 mice resulted in an intermediate response with 41% of the progeny developing ANoAs (9). Other gene(s) outside the MHC have also been shown to affect susceptibility. The influence of these background genes was observed when comparing mice of the H-2^s haplotype on an A background to those on a B10 or a B6 background (5,9). Non-MHC genes on the A background enhanced susceptibility while those present on the B background decreased responsiveness. Within the B background, B6 were more suppressive than B10.

Not all of the effects seen with $HgCl_2$ administration are as tightly controlled by the MHC as the ANoA response. For example, A/J mice (H-2^k) did develop antichromatin (ACA) and antihistone (AHA) antibodies despite being resistant to ANoA induction (7). The immune complex deposition seen in this model also does not appear to be under the influence of the H-2 complex. Balb/c (H-2^d) mice developed renal mesangial and vessel wall immune complexes despite being completely resistant to ANoA induction (9,10). When only ANoA susceptible mice (H-$2^{s,f,q,s'}$) were examined for renal IgG

deposition the results were similar in that only the H-2^s mice showed significant immune complex deposition (11,12).

Tissue concentrations of mercury chloride has been examined in several strains of inbred mice (13). Most $HgCl_2$ accumulates in the kidney primarily, with the liver being the second largest pool followed by the gastrointestinal tract, skin, spleen and testicles. The levels reached a steady state in the blood and liver by 4 weeks with the spleen and liver reaching steady state at 8 weeks. Analysis of thymi revealed steadily increasing concentrations throughout the 12 week treatment period. Interestingly, the authors also noted that when comparing the H-2-congenic susceptible B10.S (H-2^s) and resistant B10.D2 (H-2^d) a correlation was detected between susceptibility to ANoA and accumulation of Hg^{2+} within the spleen (13). Autometallography has revealed that within the tissues of the immune system mercury chloride accumulated in macrophages but was not found in non-phagocytic antigen presenting cells (follicular dendritic cells, interdigitating cells and B cells). At the subcellular level mercury was localized in the lysosomes of the macrophages (14).

Role of T Cells

The requirement for T cells in this model was shown by Hultman *et al.* using the nude (athymic) mutation (15). SJL/N mice homozygous for the nude mutation (*nu/nu*) failed to develop neither the ANoA nor the systemic immune complex deposits seen in littermates heterozygous for the nude mutation (*nu/+*). Furthermore, the specific subset of T cells required was elucidated by noting a lack of response in euthymic SJL/N mice given anti-CD4 monoclonal antibody in addition to the mercury chloride treatment.

When *in vivo* T cell responses to $HgCl_2$ were studied using flow cytometry in susceptible A.SW mice, a long lasting increase in the number of T cells dominated by the $CD4^+$ subset was observed which was absent in resistant A.TH mice (16). In contrast, van Vliet *et al.* (17) reported that both H-2^s and H-2^d mice expressed enhanced splenic numbers of T cells expressing the activation marker $CD45RB^{lo}CD4^+$.

Similar effects were seen when the ability of mercury to stimulate cells *in vitro* was studied. *In vitro* treatment of lymphocytes with $HgCl_2$ resulted in a significant increase in DNA synthesis in A.SW mice while only minimal increases were seen in DBA/2 mice (5). Anti-CD4 antibody again completely abrogated the response suggesting the importance of T helper cells in this model. Interestingly, mercury-exposed lymphocytes from the low responder mice became high responders, as measured by [^{3}H]Thymidine uptake, after the removal of excess mercury by washing (18). This activation of T cells *in vitro* led Jiang *et al.*(19) to examine whether mercury acted as a T cell mitogen or as a superantigen. They found a biased usage of TCR V_β subsets by $CD4^+$ cells which they interpreted as a superantigen effect.

Cytokines

Considering the effects mercury has on T cells, cytokines must play an important role in $HgCl_2$-induced autoimmunity. *In vitro*, the continuous presence of mercury induced interleukin-2 (IL-2) and interferon-γ (γ-IFN) but not IL-4 production from both high and low responder mice. In contrast, pretreating the cells with mercury and then washing it away resulted in production of IL-4 from both groups (18).

Cytokine profiles of mice treated with mercury has given somewhat conflicting results. An analysis of IL-4 mRNA from two H-2-congenic strains revealed a strong increase in production in splenic $CD4^+$ T cells of the $H\text{-}2^s$ mice while those of $H\text{-}2^d$ mice showed only a weak increase (17). This result, along with similar results seen in the rat model of $HgCl_2$-induced autoimmunity (20,21), led Goldman *et al.* (22) to propose that susceptibility relies on the initiation of a TH2 response while resistance correlates predominantly with a TH1 response.

Further evidence of this dichotomy was provided by the fact that $HgCl_2$-induced a striking increase, up to 30-fold, of the level of IgE in susceptible A.SW but not in resistant B6, DBA/2 or B10.D2 mice (17,23). The serum IgE levels are important because they, as well as, the increases seen in class II expression are both highly dependent on the TH2 cytokine IL-4 (24). The connection between IgE and IL-4 was further strengthened in this model by Ochel *et al.* (25) who showed that treating susceptible A.SW mice with anti-IL-4 antibody completely abrogated the increase in total IgE. Surprisingly, this treatment did not prevent ANoA production although it did influence the pattern of IgG subclass distribution.

Using antibody response to sheep erythrocytes (sRBC) in mercury treated H-2-congenic strains Doth *et al.* (26) also proposed a preferential activation of either TH1 or TH2 cells based on haplotype. The response to sRBC antigens is normal in treated B10.S mice while it is depressed in B10.D2 animals. They showed, using monoclonal antibodies against γ-IFN, that the suppression seen in B10.D2 mice was mediated by γ-IFN, a TH1 cytokine. Furthermore, treatment of B10.S mice with recombinant γ-IFN suppressed their

response to the sRBC antigens, yet did not prevent the induction of ANoAs nor the immune complex glomerulonephritis.

An argument against the TH1/TH2 dichotomy in this model was proposed by Johansson *et al.* (16). They found that A.SW mice showed a modest increase of γ-IFN and IL-4 producing cells while H-2-congenic A.TH mice showed no increase in cytokine producing cells. Remarkably, the susceptible SJL strain, despite being severely deficient in TH2-promoting $CD4^+$, $NK1.1^+$ T cells, increased their number of γ-IFN producing cells. This indicated that a predominantly TH2 response is not necessary for the induction of autoimmunity by mercury.

Recent studies conducted by Pollard *et al.* (27) provided the strongest evidence to date against the idea that a TH1 response corresponds with resistance while a TH2 response is required for autoimmunity induction. Using $H\text{-}2^s$ mice deficient by gene knockout for IL-4 and γ-IFN, they showed that all of the features of autoimmunity are controlled by γ-IFN, a TH1 cytokine. $IL\text{-}4^{-/-}$ mice did develop ANoAs while $\gamma\text{-}IFN^{-/-}$ were completely resistant. They concluded that the requirement for γ-IFN suggests that antigen dose is the limiting factor in autoimmunity induction.

The idea of a dose response relationship was examined by Hultman *et al.* (28). They found a positive correlation between the dose of $HgCl_2$, the total body burden of mercury, and the degree of autoimmunity expressed as the serum ANoA titer. The minimum observed adverse effect level (MOAEL) was 1.25 mg $HgCl_2$ / liter drinking water (1.25 ppm). At this dose approximately 50% of the mice responded. No ANoAs were seen in mice given 0.625 ppm while 100% responded to 5 ppm. They estimated,

taking into account the absorption of inorganic mercury by the gastrointestinal tract, that the MOAEL is 7-14 μg Hg/ kg bodyweight. This is much less than the standard dose of 1.6 mg/kg used in the literature.

B Cells / Antibodies / MHC

Mercury also has pronounced effects on B cells as evidenced by the increases seen in immunoglobulin and class II surface expression. For example, SJL mice developed splenic cell hyperplasia with a transiently increased number of cells secreting IgM, IgG and IgG1 immunoglobulins, as well as increased serum immunoglobulin concentrations. Resistant B6 mice also showed similar increases although they were more short lived (29). A significant increase in anti-TNP producing cells seen in SJL mice but not in B6 mice provided evidence for polyclonal B cell activation in the former. H-2-congenic strains also show the differential effects on B cells of mercury administration. B10.S mice showed significantly increased numbers of Ig producing splenic B cells of the IgG1 (30-fold), IgG2a (7-fold) and IgE (5-fold) while B10.D2 mice in contrast did not show any significant increases (17).

Mercury also enhances class II expression on B cells from both ANoA resistant B10.D2 and ANoA susceptible B10.S mice although significantly higher levels were observed in the B10.S mice (17). Treatment with an anti-IL-4 antibody completely prevented the increases seen in both strains. In addition to mercury influencing class II expression, class II molecules are required for some of the effects induced by mercury. In an *in vitro* system using monoclonal antibodies against class II, Hu *et al.* (30) were able to

completely abrogate the cytokine production and proliferation induced by mercury. The requirement for class II molecules for the induction of ANoAs *in vivo* has not been investigated. In chapter 2, we formally address this question using mice whose MHC I-A gene has been deleted.

Fibrillarin

The nucleolar autoantigen against which $HgCl_2$-induced autoantibodies are directed has been identified as fibrillarin, a 34 kDa U3 ribonucleoprotein involved in the first step of preribosomal RNA processing (31,32). It is interesting that up to 58% of human scleroderma patients spontaneously produce autoantibodies against the same U3 ribonucleoprotein (33,34). Several groups have compared the reactivities toward fibrillarin of mercury-induced ANoAs with human scleroderma sera and found that they were indistinguishable (22,34). Both groups of autoantibodies recognize the full length protein but lose reactivity, in all but the highest titrated human sera, if either the N- or C-regions were cleaved (35).

In an attempt to determine what the T cell antigen is, bulk T cells from $HgCl_2$-treated B10.S mice were challenged with a variety of different self proteins that contained minute amounts of mercury. The T cells reacted to all the proteins in an anamnestic fashion (36). Bulk T cells obtained after one week of treatment also reacted anamnestically to Hg-complexed fibrillarin, whereas after eight weeks a T cell response against untreated fibrillarin predominated. These results suggested determinant spreading of T cell specificity. Consistent with this, analysis of T cell hybridomas established from

mercury-treated H-2^s mice revealed two types of CD4$^+$ T cells: one that specifically recognized Hg-complexed fibrillarin and another that reacted to untreated fibrillarin (37). Interestingly, both the Hg-induced and native fibrillarin determinants were presented without the addition of fibrillarin when spleen cells from animals treated with mercury were used as antigen presenting cells. In contrast, spleen cells from untreated mice could activate fibrillarin-specific T cells only if exogenous fibrillarin was added. This indicated that $HgCl_2$ can induce abnormal presentation by antigen presenting cells of a self protein.

In an attempt to understand the mechanism whereby mercury elicits an autoantibody response that specifically targets fibrillarin, Pollard *et al.* (38) studied the antigenicity and molecular properties of fibrillarin from cells undergoing mercury-induced death. Under nonreducing SDS-PAGE conditions, fibrillarin from mercury treated nuclei showed aberrant migration as evidenced by its change in migration from 34 kDa to 32 kDa. This modified fibrillarin also lost its B cell antigenicity as measured by both indirect immunofluorescence and immunoprecipitation. If either one or both of the two cysteines which fibrillarin contains were mutated to an alanine, the aberrant migration was abolished. This combined with the fact that iodoacetamide, which alkylates cysteine residues, also abolished the aberrant migration led the authors to propose that fibrillarin forms a disulfide bond which then appears as the 32 kDa band. They concluded that unmodified fibrillarin is the B cell antigen and mercury-modified fibrillarin is the T cell antigen.

The mechanism whereby mercury can induce autoimmune disease is a complicated one. To gain a better understanding of the genetics behind the response we first

investigated whether the class II I-A molecule itself or a closely linked gene(s) was a requisite for susceptibility. We also sought to determine what effects, if any, the other class II molecule, I-E, had on susceptibility. In our initial studies we unexpectedly found that susceptibility was not codominantly inherited as previously reported. This led us to perform adoptive transfer experiments to elucidate the mechanisms whereby the resistant haplotype could down-regulate the response. In addition, we used these chimeric mice to investigate the cellular interactions between B and T cells in this model.

CHAPTER 2
CLASS II HAPLOTYPE DIFFERENTIALLY REGULATES IMMUNE RESPONSE IN $HgCl_2$-TREATED MICE

Introduction

Subtoxic doses of $HgCl_2$ in animals induce an autoimmune disease characterized by autoantibody production and variable amounts of immune complex glomerulopathy (22). In mice, for example, the glomerulopathy is relatively mild and characterized by mesangial deposits of IgG and C3 (10). Other features, such as splenic hyperplasia with increased serum immunoglobulins (especially IgG and IgE) have also been described (23,29). However, the most striking finding in mice is the antinucleolar antibodies (ANoA) elicited in susceptible strains as soon as one week after the start of treatment (4). The principal nucleolar autoantigen has been identified as fibrillarin, a U3 ribonucleoprotein involved in the first step of preribosomsal RNA processing (31,39). Interestingly, this same specificity is seen in patients suffering from systemic sclerosis and other connective tissue disorders (33,34). Even more remarkably, a comparison of the specificity of antifibrillarin autoantibodies between human and murine origin showed similar, if not identical conformational epitopes (40).

The mechanism by which $HgCl_2$ causes autoimmunity is unknown, but the fact that a human scleroderma-associated autoantibody specificity can be produced and that certain

toxic exposures have resulted in scleroderma-like illnesses (41,42) makes it an important and intriguing model. Both MHC class II-linked loci and unknown non-MHC loci govern susceptibility, with mice of the H-2^s haplotype being high responders, while those of the H-2^b haplotype are resistant or low responders (5). Non-H-2 genes in H-2^s mice did not prevent the disease but had a pronounced effect on the antinucleolar antibody (ANoA) titers (12). By using intra-H-2 recombinants, it has been shown that responsiveness could be mapped to the I-A region of the MHC. The other class II locus, I-E, was shown to either suppress (6) or have no effect on the immune response (7). The class II-linked susceptibility gene has previously been reported to be dominant, although the disease was attenuated in F_1 animals when compared to homozygous animals (5,9,43). The fact that most of these studies used non-MHC-congenic strains, however, makes it difficult to interpret the effects of non-MHC genes and I-E expression on the ANoA response.

To investigate the control of the ANoA response by the I-A region to $HgCl_2$ in the absence of non-MHC loci differences we studied two MHC-congenic strains of mice on the B6 background. In addition to the susceptible H-2^s haplotype, we chose to study H-2^b rather than the more commonly used H-2^d haplotype because of the availability of animals on the B6 background which do not express class II due to a targeted disruption of the I-$A_\beta{}^b$ chain. In addition, the H-2^s and H-2^b haplotypes have both lost functional expression of I-E, allowing the effects of I-A and I-E to be isolated. In our initial studies, we unexpectedly found that F_1 progeny between B6.SJL (H-2^s) and B6 (H-2^b) mice were highly resistant to $HgCl_2$ treatment and suggested that this might have been due to a dominant negative gene linked to I-A^b. Such a possibility is not without precedent.

Recently, the clinical manifestation of arthritis in the autoimmune model of collagen-induced arthritis was shown not to be exclusively limited to a particular MHC class II antigen but may be due to linked genes which participate in intracellular loading and selection of antigenic peptides (44). To distinguish between these two possibilities we generated mice on the B6 background which expressed I-A^s and carried the genes linked to the I-A^b region but did not express I-A^b. These mice were as susceptible to the induction of ANoA as H-2^s homozygous mice, despite carrying the genes linked to I-A^b. We also examined the effects of the other class II molecule, I-E, in our system by developing B6.SJL mice which carried the I-$E_\alpha{}^d$ transgene (45), allowing the functional expression of I-E on the H-2^s background. Contrary to a previously published report and to our experience with I-A, the expression of I-E did not alter the ANoA response. Thus, our experiments demonstrate that it is the expression of the resistant H-2 allele (I-A^b) itself that down-regulated the ANoA response of F_1 crosses between resistant and sensitive haplotypes and strongly suggest that it is the H-2^s molecule itself that is specifically required. These results have important implications for understanding the mechanism by which $HgCl_2$ exposure causes a specific autoimmune syndrome.

Materials and Methods

Mice

C57BL/6J (B6; I-A^b, I-E^-, Igh^b), C57BL/6J.SJL-*H-2*^{s} (B6.SJL; I-A^s, I-E^-, Igh^b), C57BL/6J-*Igh*^{a} *Thy1*^{a} *Gpi*^{a} (B6.TC; I-A^b, I-E^-, Igh^a), A.BY/SnJ, A.SW/SnJ, SJL/J and Balb/cJ mice were obtained directly or from breeders purchased from The Jackson

Laboratory (Bar Harbor, ME). Tg(H-2I:Eα)Bri39 (B6.I-$E_\alpha{}^d$) mice were a gift from R. L. Brinster (45). $CD2^{TM}$ (MHC Class II deficient, B6 background) were purchased from Taconic (Germantown, NY). These mice lack any cell surface expression of I-A due to the insertion of a loss of function mutation into the I-$A_\beta{}^b$ gene (46). B6.SJL.I-$E_\alpha{}^d$ mice were developed by crossing B6.SJL with B6.I-E_αd and then backcrossing to B6.SJL. The B6.SJL.I-$E_\alpha{}^d$ mice were then selected using FACS analysis. These mice expressed I-E molecules consisting of a β-chain of the b allele and an α-chain of the d allele (47,48). B6.I-$A^{g/b}$ and B6.I-$A^{g/-}$ mice were developed by intercrossing $CD2^{TM}$(B6.I-$A^{-/-}$) with B6 and the resultant F_1 bred to B6.SJL. The MHC class II expression was verified using FACS analysis. To rule out an allotype effect on susceptibility B6.SJL.Igh^a mice were developed by intercrossing B6.SJL and(B6.SJL x B6.TC)F_1. FACS analysis was used to select progeny which were I-A^s and Igh$^{a/b}$. These mice were then intercrossed and the progeny selected which were B6.SJL.Igh^a using FACS and the strain was established from a single breeding pair. All mice were housed in AAALAC approved facilities in compliance with all applicable federal, state and local laws (Table 2-1).

$HgCl_2$ Treatment

$HgCl_2$ (Sigma Chemical Co., St. Louis, MO) was prepared in sterile, pyrogen-free phosphate buffered saline (PBS). Mice were injected subcutaneously at a dose of 1.5 mg/kg three times weekly unless noted otherwise.

Indirect Immunofluorescence

Sera from mice was tested for the presence of ANoA by indirect immunofluorescence using commercially prepared mouse frozen kidney slides (Sanofi, Chaska, MN). The slides were incubated with sera diluted 1:50 in PBS for 30 minutes at room temperature. For nonallotype-specific ANoA. the slides were incubated for 30 min at room temperature with FITC-conjugated goat anti-mouse IgG (Fc fragment specific, Jackson ImmunoResearch, West Grove, PA) diluted 1:50 in PBS. For the allotype-specific ANoA determination slides, were incubated with rabbit anti-mouse $IgG2a^a$ or $IgG2a^b$ (Nordic Labs, Capistrano Beach, CA) followed by FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). These reagents had been pre-titrated by ELISA against an allotype-nonspecific rabbit anti-IgG2a antibody (Nordic Labs) to produce equivalent sensitivities. Antinucleolar staining was evidenced by an intense homogeneous staining of the nucleoli (Fig. 2-1).

Antibodies

MK-S4 (HB4; murine IgG_{2b} anti-I-$A_\beta^{s,f,u}$ (49)) and 14-4-4S (HB32; murine IgG_{2a} κ anti-I-$E_\alpha^{k,d}$ (50)) were purchased from American Type Tissue Culture (Rockville, MD). D3-137.5 (murine IgG_{2a} anti-I-A_β^b antigen (51)) was originally obtained from Tonkonogy. D77, originally developed against yeast fibrillarin but cross-reactive with fibrillarin of rat and human origin (52), was a kind gift of Dr. John Aris (University of Florida, Gainesville, FL). Aliquots of stock mAb were prepared from overgrown cell culture supernatant

which was affinity purified on a protein G column. Monoclonal antibodies were labeled either with biotin hydrazide (53) or fluorescein isothiocyanate (FITC) (54), as needed.

Immunizations

Mice were immunized by an intraperitoneal injection of 50 μg of hen egg lysozyme (HEL; Sigma Chemical Co., St. Louis, MO) in 100 μl of CFA (Difco Laboratories, Detroit, MI) diluted 1:1 with sterile PBS. Fourteen days later the mice were boosted with the same antigen in IFA. Mice were then bled via tail vein 8 weeks after the secondary immunization and the sera stored at -20°C until analyzed by ELISA.

Anti-HEL ELISA

Determination of antigen-specific antibody levels were determined using an indirect ELISA. Immulon® 2 microtiter plates (Dynatech Laboratories, Inc., Chantilly, VA) were coated overnight at 4°C with HEL at 10μg/ml in bicarbonate buffer (0.1M, pH 8.2). Between all incubation steps microplates were washed three times with borate-buffered saline (BBS: 25 mM $Na_2B_4O_7 \cdot 10\ H_2O$, 75 mM NaCl, 100 mM H_3BO_3, pH 8.4) containing 0.05% Tween 20. After blocking with BBS containing 0.5% BSA and 0.4% Tween 20, serial dilutions of serum samples diluted in the blocking solution were incubated overnight at 4°C. Either biotinylated goat anti-mouse IgG (Fc fragment specific) or biotinylated goat anti-mouse IgM (mu chain specific)(Jackson Immunoresearch, West Grove, PA) was added for one hour at room temperature. The indicator system consisted of ExtrAvidin® alkaline phosphatase (Sigma Chemical Co., St.

Louis, MO) and the substrate Sigma 104® phosphatase substrate (p-nitrophenol phosphate, disodium, hexahydrate). The substrate turnover was determined by the difference between the OD_{405} and OD_{620} on a BioTek Instruments, Inc. (Winoski, VT) Ceres 9000 microplate reader. The concentration of antigen-specific IgG and IgM is reported in equivalent dilution factors of standardized reference B6.SJL sera. This is defined by the formula: EDF = (dilution of a standard reference sera that gives the equivalent OD of the test serum) X 10^4.

Flow Cytometry

Approximately 200 µl of tail vein blood was collected into heparinized tubes. PBMCs were isolated using Lympholyte M (Cerdarlane, Hornby, Ontario, Canada) density gradients. The cells were then collected into PBS, supplemented with 3% FCS and 0.1% NaN_3. For cell surface staining, saturating amounts of 14-4-4S , D3-137.5-biotin or MK-S4-FITC were used as the first step. The second step consisted of incubating with FITC-conjugated goat anti-mouse IgG (Fc fragment specific) or phycoerythrin (PE)-conjugated streptavidin (Jackson Immunoresearch, West Grove, PA). The cells were then washed three times in PBS and fixed with an equal volume of 2% paraformaldehyde. List mode data were acquired on a FACScan flow cytometer (Becton Dickinson, Palo Alto, CA) using PC Lysis software. Dead cells were excluded by forward and side scatter gating. List mode files were then analyzed using Lysis II software.

Statistical Analysis

The difference between the number of mice showing an ANoA between B6.I-$A^{s/b}$ and B6.I-$A^{s/-}$ was analyzed using Fischers exact test. A t-test was used to compare baseline I-A^s MFI (mean fluorescent intensity) values between these two groups. Differences between MFIs of the other groups was tested by an ANOVA (analysis of variance) followed by Dunnett's multiple comparison procedure. A p value of <0.05 was considered to be significant.

Results

F_1 Mice Between a Resistant and Sensitive Haplotype Were Resistant to ANoA Induction

In the course of our initial experiments with the $HgCl_2$ model of induced autoimmunity, we examined the ANoA response in an F_1 cross between the susceptible B6.SJL (H-2^s; Igh^b) and the resistant B6.TC (H-2^b: Igh^a) strains. As expected, B6.SJL (n=18) and B6.TC (n=13) were 100% and 0% susceptible, respectively. A representative example is shown in Fig. 2-1, where a serum sample from a B6.SJL mouse treated for 5 weeks with $HgCl_2$ shows a distinct nucleolar pattern. However, the vast majority (42/45) of F_1 intercrosses between these two strains were also resistant to $HgCl_2$ treatment (Table 2-1). The resistance of the F_1 mice was surprising. Although the exact mechanism for $HgCl_2$-induced autoimmunity is unknown, the fact that the class II molecules are co-dominantly expressed led us to expect that the expression of I-A^s would be sufficient to confer susceptibility. To make sure this resistance was not due to the allotype difference,

B6.SJL.Igh^a (H-2^s, Igh^a) mice were produced and found to be 100% susceptible to ANoA formation. This showed that the Igh locus did not have an effect on susceptibility. Finally, to rule out a dose effect in the F_1 crosses, groups of 4-6 (B6.SJL x B6.TC)F_1 were dosed with either 3.0, 4.5, 7.5 or 10 mg/kg of $HgCl_2$ for 3 weeks with no mice developing an ANoA titer (data not shown).

Because it has been reported that the B6 background produces lower titer ANoA responses than other H-2^s strains, other crosses were tested. Initially, mice on the A background were used. Consistent with previous studies, A.SW (H-2^s) mice were 100% (3/3) susceptible while A.BY (H-2^b) mice were resistant (0/4). Next, F_1 crosses between these two strains were generated. Despite the more susceptible A background, only twenty percent (1/5) of (A.BY X A.SW)F_1 mice showed ANoA positivity after treatment. To rule out a maternal effect in F_1 mice as previously suggested (9), the same strain was produced except that the A.SW strain was used as the female parent. This cross, (A.SW x A.BY)F_1, resulted in only 11% (1/9) of the animals responding. In further attempts to find an F_1 cross between resistant (H-2^b) and sensitive (H-2^s) mice in which the susceptibility gene(s) was dominant, the following crosses were tested: (A.SW X B6)F_1, (B6 X SJL/J)F_1 and (SJL/J X B6)F_1. None of these animals developed an ANoA titer after mercury treatment.

The specificity of the autoantibody response was verified by comparing an immunoblot of sera from a B6.SJL mouse. This serum sample which reacted with a 34-kDa nucleolar protein shown to be fibrillarin by mAb D77 (data not shown).

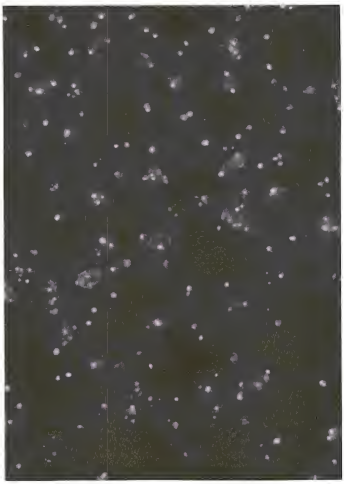

Figure 2-1 Serum from a B6.SJL mouse treated for 5 weeks with $HgCl_2$ on mouse frozen kidney slides and stained with FITC-conjugated goat anti-mouse IgG as a second step showing intense staining of nucleoli.

Table 2-1 Congenic and F_1 mice: serum ANoA[a] after 5 weeks of treatment with $HgCl_2$[b]

Strain	Class II	I-A		I-E[c]		IgG1	n[d]	%positive[e]
	Haplotype	A_β	A_α	E_β	E_α	allotype		
A.BY	b	b	b	(b	b)	a	4	0
A.SW	s	s	s	(s	s)	a	3	100
(A.BY x A.SW)F_1	s/b	s/b	s/b	(s/b	s/b)	a	5	20
(A.SW x A.BY)F_1	s/b	s/b	s/b	(s/b	s/b)	a	9	11
(A.SW x B6)F_1	s/b	s/b	s/b	(s/b	s/b)	a/b	8	0
(B6 x SJL/J)F_1	s/b	s/b	s/b	(s/b	s/b)	b	6	0
(SJL/J x B6)F_1	s/b	s/b	s/b	(s/b	s/b)	b	6	0
B6.SJL	s	s	s	(s	s)	b	18	100
B6.TC	b	b	b	(b	b)	a	13	0
(B6.SJL x B6.TC)F_1	s/b	s/b	s/b	(s/b	s/b)	a/b	45	7
B6.SJL.Igh[a]	s	s	s	(s	s)	a	9	100

[a]ANoA, antinucleolar antibodies
[b]1.5 mg $HgCl_2$/kg s.c. three times per week
[c]alleles written in parenthesis denote nonexpression on the cell surface of the $E_\beta E_\alpha$ molecule
[d]Number of mice
[e]Fraction of positive mice

The A^b Gene Product Conferred Resistance in F_1 Mice

Because of the marked resistance of all (s/b) F_1 mice tested and the possibility that this effect was being mediated by non-class II molecules, the role of class II was tested directly. Taking advantage of the availability of B6 mice with targeted deletions of the I-$A_\beta{}^b$ gene, mice heterozygous for this induced mutation were mated with B6.SJL mice to generate two groups of mice within the littermates. One group possessed the genes linked to I-A^b without expressing the molecule itself, while the other possessed these same genes but also expressed I-A^b. Those mice that expressed I-A^b (I-$A^{s/b}$) were resistant (1/14) to the development of ANoA, as expected from our earlier results, despite expressing the susceptible I-A^s molecule. However, those that possessed both I-A^s and I-A^b-linked genes, but not I-A^b expression itself (I-$A^{s/-}$), were as susceptible (17/20) to ANoA

formation as B6.SJL homozygous mice (Table 2-2). This clearly shows that it is the I-A^b molecules themselves and not a linked gene which conferred resistance. As a negative control, no mice (0/8) developed ANoA after treatment with PBS (Table 2-2).

Table 2-2 Effect of A_β^b deletion on ANoA[a] in $HgCl_2$-treated(B6.SJL x B6.I-$A^{b/-}$) F_1 mice

Class II	n[c]		%positive[d]	
Haplotype[b]	$HgCl_2$[e]	NaCl[f]	$HgCl_2$	NaCl
s/b	14	4	7[g]	0
s/-	20	4	85	0

[a]ANoA, antinucleolar antibodies measured after 5 weeks of treatment
[b]F_1 mice either did (s/b) or did not (s/-) express I-A^b but were heterozygous at all the MHC loci
[c]Number of mice
[d]Fraction of positive mice
[e]1.5 mg $HgCl_2$/kg s.c. three times per week
[f]0.1 ml NaCl s.c. three times per week
[g]Significant difference between (s/b) and (s/-) mice ($p < 0.05$ using Fischer exact test)

To rule out the possibility that the differential susceptibility between I-$A^{s/b}$ and I-$A^{s/-}$ was due solely to increased expression of I-A^s and not a negative regulatory influence by I-A^b, we examined I-A levels by haplotype-specific flow cytometry. Baseline I-A^s expression was similar whether or not the mouse carried the disrupted gene (MFI = 45 vs. 49; $p = 0.1185$). MHC class II expression on PBMCs was similarly increased in both strains when treated with $HgCl_2$ (Fig. 2-2). I-A^s expression was increased by 44% ($p<0.05$) in the mice which carried the disrupted allele, while it was increased by 48% ($p<0.05$) in those mice which expressed both haplotypes (s/b). The increase in I-A^b expression in the mice which carried that gene was 48% ($p<0.05$). No significant increase in class II expression was seen in those animals treated with PBS.

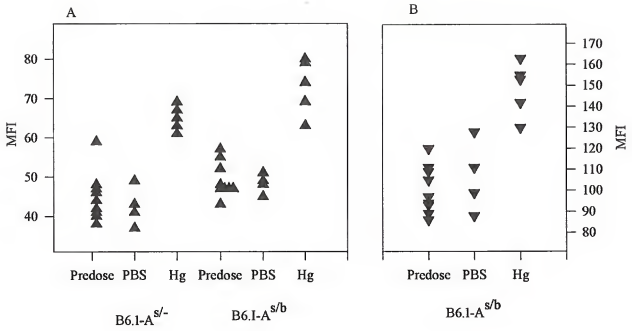

Figure 2-2 Effect of $HgCl_2$ on class II expression in(B6.SJL x B6.I-$A^{b/-}$)F_1 mice. (A) Predose I-A^s expression was similar in B6.I-$A^{s/b}$ and B6.I-$A^{s/-}$ mice. I-A^s expression was increased by 44% in the mice which carried the disrupted allele, while it was increased by 48% in those mice which expressed both haplotypes (s/b). (B) The increase in I-A^b expression in the mice which carried that gene was 48%. No significant increase in class II expression was seen in those animals treated with PBS. *$p \leq 0.05$

Expression of I-E Had No Effect on Susceptibility to $HgCl_2$

It has previously been reported in a number of autoimmune models that I-E expression can down-regulate autoantibody titers. Intra-MHC recombinant strains have given conflicting results in the $HgCl_2$ model, but interpretation has been hampered by the difficulty in isolating the effects of I-E expression. To test this more explicitly and to see if competing expression by another class II molecule is a general property in the down-regulation of the anti-nucleolar response to mercury, B6.SJL mice expressing the I-E_α^d transgene were bred. Restoring the ability to express I-E in otherwise susceptible mice had no effect on the ability to respond to $HgCl_2$. All (5/5) B6.SJL.I-E_α^d mice retained the ability to generate an ANoA titer upon $HgCl_2$ treatment (Table 2-3). Mean I-E expression in these mice (MFI = 280.7) was similar to Balb/c mice (MFI = 282.1) which normally express the I-E gene product. I-A^s expression between B6.SJL and B6.SJL.I-E_α^d mice was also similar (MFI = 267.9 vs. 253.2) (Fig. 2-3). The breeding scheme used to generate the B6.SJL.I-E_α^d mice also produced (B6.SJL x B6.I-E_α^d)BC_1 mice which either did (n=14) or did not (n=13) express the transgene. It has been suggested in the BxSB autoimmune model that I-E expression could block autoantibody production by producing an I-E-derived peptide that binds to I-A^b with such affinity that it competes with the putative autoantigen-derived peptides for presentation (6). Starting with the same transgenic parent strain as was used in the present experiments, we have also confirmed this observation in the BxSB model (unpublished data). If this is a general property in autoimmunity, it might be expected that I-E expression would convert an otherwise

resistant $HgCl_2$-treated s/b heterozygous mouse into a sensitive one. Despite normal levels of expression of I-E, however, all of these mice were resistant (Table 2-1).

Table 2-3 Effect of I-E on ANoA[a] response

Strain	Class II Haplotype	A		I-E Expression	n[b]	%positive[c]
		A_β	A_α			
B6.SJL.I-E_α^d	s	s	s	+	5	100
(B6.SJL x B6.I-E_α^d)F_1	s/b	s/b	s/b	+	14	0
(B6.SJL x B6)F_1 [d]	s/b	s/b	s/b	-	13	0

[a]ANoA, antinucleolar antibodies measured after 5 weeks of treatment with 1.5 mg $HgCl_2$/kg s.c. three times per week
[b]Number of mice
[c]Fraction of positive mice
[d]Non-transgenic littermate of(B6.SJL x B6.I-E_α^d)F_1

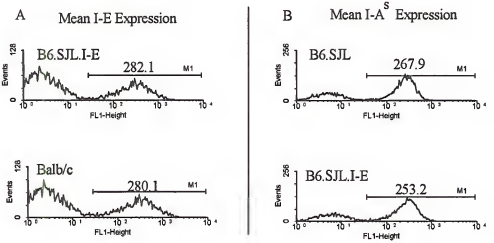

Figure 2-3 Class II expression in B6.SJL.I-E_α^d mice. (A) Mean I-E expression was similar to Balb/c mice. (B) Mean I-A^s expression was not affected by I-E expression. Single-parameter fluorescence histograms. Increasing fluorescence intensity is plotted on the x-axis in log fluorescent units versus cell number on the y-axis The numbers represent the MFI (mean fluorescent intensity) of the cells expressing the antigen of interest.

Responsiveness to Exogenous Antigen Was Inherited Co-dominantly in F_1 Mice

The lack of ANoA's in our F_1 mice led us to evaluate their responses to an exogenous antigen (HEL). As expected, mice of the b haplotype (B6.TC) responded poorly while the B6.SJL mice mounted an intermediate titer. An intermediate response was seen in the F_1 cross (Figure 2-4). Balb/c mice responded with a titer approximately 10 fold higher than any of the mice on the B6 background (data not shown). Thus it appears that the responsiveness in our F_1 mice to HEL was inherited in a co-dominant fashion.

Discussion

The mercury chloride response in rodents is a complicated one and classification of responder and non-responder strains depends on the outcome being measured. For example, by ANoA titer, the b haplotype is a non-responder. However, by measuring the weights of draining popliteal lymph nodes of (DBA/2 X C57BL/10)F_1 mice exposed to $HgCl_2$, b haplotype has been classified as intermediate responders.

The anti-fibrillarin response by susceptible mice, however, is very reproducible and specific and therefore more amenable to mechanistic dissection. As a first step, it is crucial to convincingly demonstrate that the anti-fibrillarin response is a property of the I-A molecule itself and not a closely linked gene. Results in the literature thus far have been suggestive but not conclusive. Robinson *et al.*(5) used two unrelated strains (A.SW and C57BL/10) to generate the F_1's. These mice were 57-100% susceptible to developing

ANoA, albeit of a lower titer. This led the authors to conclude that the response was controlled by the H-2 region and one or more unlinked genes. Recently, Hultman *et al.* (9) found that 75% of male and 11% of female mercury treated (SJL x B6)F_1 mice showed a clumpy IgG ANoA pattern with a low titer. These differences were attributed to the non-H-2 genes. For example, the A genetic background seemed to enhance susceptibility to the $HgCl_2$ treatment, while the B10 background seems to strengthen the resistance. They also suggested that the B6 non-H-2 background genes are more suppressive on ANoA expression than equivalent genes from the B10 strain. A similar effect of non-H-2 genes was seen by Hultman *et al.* (12) when comparing the ANoA response in several H-2^s strains. B10.S mice showed a much lower titer than either A.SW or SJL mice after $HgCl_2$ treatment. This was again attributed to the non-H-2 genes.

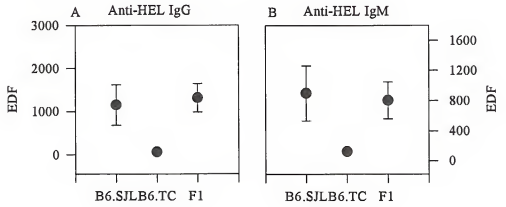

Figure 2-4 Anti-HEL antibody levels 10 weeks after immunization. The response to HEL was inherited co-dominantly in(B6.SJL X B6.TC)F_1 mice. B6.SJL and (B6.SJL X B6.TC)F_1 mice respond to HEL while B6.TC do not. (A) IgG (B) IgM; EDF +/- SEM

In the process of studying the cellular control of the mercury-induced anti-fibrillarin response, we examined the susceptibility of a number of F_1 mice in our own laboratory. Contrary to previously published results summarized above, our F_1 mice seldom produced a measurable ANoA titer most likely due to the strong suppressive effects seen when using mice on the B6 background. This lead us to re-examine the role of class II expression. Several genes linked to class II have been shown to participate in antigen processing and presentation (44,55,56). The possibility existed that the genes linked to the I-A^b molecule could be altering the processing and/or presentation of fibrillarin resulting in a peptide which no longer is immunogenic. If this were the case then mice which express I-A^s and carry the I-A^b linked genes without displaying the I-A^b molecule itself would be expected to be resistant.

Since the ANoA response was not dominant in our F_1 mice we compared the responses to HEL in which responsiveness should be inherited in a co-dominant fashion. Mice of the b and s haplotype are classified as non-responders to HEL when measured by PFC assays, although SJL (H-2^s) mice do mount an intermediate serum titer (57). An analysis of anti-HEL serum titers revealed that B6.TC mice responded poorly, as expected, while both while B6.SJL and (B6.SJL x B6.TC)F_1 mice were intermediate responders. It appears that there is a difference in the inheritability of responsiveness between the $HgCl_2$-induced ANoA response and that to a conventional exogenous antigen.

To investigate whether the resistance of F_1 mice is due to expression of the I-A^b molecule or a linked gene, F_1 mice on the B6 background were produced which lacked

expression of the I-A^b molecule due to disruption of the MHC class II I-$A_\beta{}^b$ gene (46). By using congenic strains which differed at only the H-2 region, we eliminated the effects of non-MHC-linked genes. Because they are of a low-responder background, the B6 strains also allowed us to more carefully isolate the effects of the MHC. Finally, the B6 background permitted us to take advantage of the availability of class II transgenics and targeted deletions. Our strategy was to cross the $CD2^{TM}$ (B6.I-$A^{-/-}$) to a wild type B6.I-$A^{b/b}$ to obtain B6.I-$A^{b/-}$ mice. These mice were then bred to a B6.SJL to obtain the two strains used for analysis: B6.I-$A^{s/b}$ and B6.I-$A^{s/-}$. The incidence of ANoA in those mice which expressed the I-A^b gene product was significantly decreased showing that the I-A^b molecule itself and not a linked gene was responsible. To rule out the possibility that the mice carrying the disrupted allele could be expressing a higher density of I-A^s molecules thus leading to the increased susceptibility, we compared the predose mean I-A^s expression between the 2 strains. The MFI of I-A^s expression for the mice carrying the disrupted allele (I-$A^{s/-}$) was not significantly different from the I-$A^{s/b}$ mice.

The possibility exists that the I-$A_\alpha{}^b$ gene product could pair with the I-$A_\beta{}^s$ molecule resulting in an haplotype-mismatched class II molecule ($A_\alpha{}^b$ / $A_\beta{}^s$). Martin *et al.*(58) showed that the inefficient assembly of haplotype-mismatched class II polypeptides results from their inability to compete with the matched pairs. Different class II MHC $\alpha\beta$ chain combinations vary greatly in their efficiency of cell-surface expression based on the allelic origin of the α and β amino-terminal regions (59). If this pair was being expressed we would have expected to see an increase in the I-A^s expression in the mice carrying the disrupted allele (I-$A^{s/-}$), since the mAb MK-S4 recognizes the β chain of I-A^s, however no difference was detected, and we believe this is an unlikely possibility.

Another possibility is that the $HgCl_2$ had a differential effect on the two class II molecules. If the $HgCl_2$ had caused a greater increase in the expression of the I-A^b molecule in those mice which expressed it, this could lead to a decreased presentation of antigen by the susceptible allele (I-A^s), perhaps falling below the threshold required for T cell activation. This is not likely since both class II molecules showed similar increases in expression upon $HgCl_2$ treatment.

To examine directly the effect of I-E expression in otherwise susceptible mice, we generated mice on a B6 background expressing the I-A^s and the I-E molecules. Like other H-2^s mice, B6.SJL mice do not express I-E due to a deletion in the I-E_α gene (60). To remedy this, we crossed B6.SJL mice with a B6 mouse carrying the I-E transgene (Tg(H-2I:Eα)Bri39) (45). After backcrossing to B6.SJL mice, the progeny which expressed I-A^s and I-E were selected. These mice remained 100% susceptible to ANoA formation despite expressing the I-E molecule. Our results do not support the observation by Mircheva *et al.* (6) that the expression of I-E suppresses the induction of ANoAs. Hultman *et al.* (7) was also not able to confirm the suppression. Both of theses studies examined the effect of I-E by using intra-H-2 recombinant strains, which meant that the conclusions were based on inference from a number of comparisons of strains. We believe that our system of using the introduction of a transgene to restore expression offers an advantage over random recombinatorial events which can carry along other genes in the process.

The possibility exists that these mice do not express I-E at physiological levels. If I-E were being expressed at excessively high levels, antigen presentation by the I-A^s

molecule could be diminished. Conversely, low-level expression of I-E could result in little physiologic effect. To rule out either of these artifacts, we compared mean I-E expression between our mice and a strain which normally expresses I-E (Balb/c). There was no difference in the mean expression between the two strains. The expression of I-A^s was also similar between B6.SJL and the B6.SJL.I-E_α^d mice. Moreover, expression of I-E was also physiologic in that it was expressed on B but not T cells (data not shown).

Merino *et al.* (47) showed that the I-E_α^d transgene protected BxSB mice against SLE. They proposed that overexpression the I-E α chain resulted in the generation of excessive amounts of a peptide displaying a high affinity for the I-A^b molecule thereby preventing autoantigen-derived peptides from being presented. This phenomenon has also been seen in mice homozygous for the *lpr* mutation (48). To investigate the possibility that this might work in reverse to promote autoimmunity in some cases, we generated the otherwise resistant (B6.SJL X B6.I-E_α^d)F_1 mice which either did or did not express I-E. Theoretically, this could block I-A^b as a functional competitor and enhance an I-A^s-mediated response. However, neither strain developed ANoA after treatment with $HgCl_2$. Thus, the expression of I-E does not promote the development of ANoA in a strain which is otherwise resistant. The results are clear, although we cannot formally exclude the possibility that a peptide derived from I-E was even more effective in blocking I-A^s. However, if this were the case then we would have expected I-E to have decreased $HgCl_2$-induced susceptibility in the B6.SJL.I-E_α^d mice.

Taken together, our results conclusively demonstrate for the first time that $HgCl_2$ responsiveness is regulated by the I-A haplotype itself and not a closely linked gene.

However, the ability to downregulate the immune response by coexpression in a resistant haplotype in an otherwise sensitive strain is a complicated one. Antigen competetion, proposed to be important in other autoimmune models (47,48), does not appear to have a dominant role in the $HgCl_2$-induced response.

CHAPTER 3
RESISTANCE TO $HgCl_2$-INDUCED AUTOIMMUNITY IN HAPLOTYPE HETEROZYGOUS MICE IS AN INTRINSIC PROPERTY OF B CELLS

Introduction

Systemic lupus erythematosus is a complicated systemic autoimmune disease with a multigenic mode of inheritance interacting with a potentially complex array of generally unknown environmental factors. Many of these features are also seen in the mercury chloride model of murine autoimmunity, which offers a unique opportunity to study the reproducible interaction between genetics and a simple, inorganic environmental agent. Susceptibility to mercury chloride in mice is characterized by splenic hyperplasia, hypergammaglobulinemia and autoantibody production (4,29), all of which appear as soon as one week after the start of treatment (4). The principle autoantibody target is fibrillarin, a U3 ribonucleoprotein found in the nucleolus and involved in the first step of preribosomal processing (31,39). The antifibrillarin specificity is also seen in patients with scleroderma and other connective tissue disorders (33,34). Recently, a comparison of the specificity of the human and murine autoantibodies showed that they recognize similar, if not identical, epitopes (40) and suggested an antigen-driven response in both.

Similar to human autoimmune disease, both MHC and non-MHC genes have been shown to have a pronounced effect on susceptibility (12). As defined by the presence of

antifibrillarin antibody, mice of the H-2^s haplotype are high responders, while those of the H-2^b and H-2^d haplotypes are nonresponders (5). In contrast, non-MHC genes affect primarily antibody titers but not specificity (12). Through the use of intra-H-2 recombinants, the presence of the $HgCl_2$-induced antifibrillarin antibody response has been mapped specifically to the I-A region (6). Previous studies in our laboratory have shown that F_1 animals between MHC-congenic susceptible H-2^s and resistant H-2^b mice to be resistant to $HgCl_2$-induced ANoAs (8). This is surprising, since the I-A molecule is co-dominantly expressed in the F_1 mice, and in other autoimmune models heterozygosity of class II either enhances autoimmunity or modestly affects antibody titers (61,62). Therefore, the profound resistance to $HgCl_2$-induced ANoAs seen in all haplotype-heterozygous mice tested raised the possibility that resistance was mediated by a dominant gene linked to I-A^b. However, further experiments demonstrated that resistance was, in fact, caused by co-expression of the I-A^b molecule itself, and that this outcome was not merely the result of lower expression of the susceptible I-A^s haplotype on the otherwise responsive B cells (8).

We have utilized adoptive transfer experiments to explore further the mechanisms by which the resistant haplotype down regulated the $HgCl_2$-induced antifibrillarin antibody response. Our experiments demonstrated that resistance was due neither to I-A^b-mediated alteration of T cell repertoire nor to inadequate I-A^s-restricted T cell help but was an intrinsic property of the resistant haplotype-heterozygous B cells. These results suggest the presence of a novel mechanism of regulation of a potentially autoreactive immune response.

Materials and Methods

Mice

C57BL/6J (B6; I-A^b, Igh^b), C57BL/6J.SJL (B6.SJL; I-A^s, Igh^b), C57BL/6J-*Igh*^{a} *Thy1*^{a} *Gpi*^{a} (B6.TC; I-A^b, Igh^a) mice were originally obtained from The Jackson Laboratory (Bar Harbor, ME) and maintained in our breeding facility.d B6.SJL.Igh^a mice were developed by intercrossing B6.SJL and(B6.SJL x B6.TC)F_1. FACS analysis was used to select progeny which were I-A^s and $Igh^{a/b}$. These mice were then intercrossed and the progeny selected which were B6.SJL.Igh^a using FACS and the strain was established from a single breeding pair. All mice were housed in AAALAC approved facilities in compliance with all applicable federal, state and local laws.

Preparation of Chimeras

Preparation of chimeras was as previously described (63). Recipient mice were provided with Septra® - (1% v/v) treated water the day prior to irradiation. On the day prior to bone marrow transfer, the mice were treated with two doses of 525 rad of γ-radiation (Gammacell 40, Atomic Energy of Canada, Ltd., Ottawa, Canada) separated by 3-4 hours. The transfer involved i.v. reconstitution with a total of 10^7 bone marrow cells from age- and sex-matched donors. Bone marrow cells were depleted of mature T cells by incubation at 4° C for 30 min with a mixture of anti-mouse T-cell serum (Cedarlane Laboratories, Hornby, Ontario, Canada), 172-4 (rat IgM anti-CD4 (64)), and 31 M (rat IgM anti-CD8 (65)) followed by treatment with C at 37°C for 1 h (Low-Tox Guinea Pig

C, Cedarlane Laboratories, Hornby, Ontario, Canada). To prevent graft rejection the B6.SJL mice receiving(B6.SJL x B6.TC)F_1 cells were given 0.1 mg i.p. of MmT1 (mouse IgG2a anti-CD90.2 (66)) at the time of transfer. 172-4 and 31 M were obtained from Dr. David Harris (University of Arizona, Tuscon, AZ) and prepared from overgrown cell culture supernatant which was affinity purified on a protein G column.

In Vivo Treatments

$HgCl_2$ (Sigma Chemical Co., St. Louis, MO) was prepared in sterile, pyrogen-free phosphate buffered saline (PBS). Mice were injected subcutaneously at a dose of 1.5 mg/kg three times weekly after graft acceptance was verified using flow cytometry. Mice were immunized intraperitoneally with 100 µg of human IgG (HGG; Sigma Chemical Co., St. Louis, MO) emulsified 1:1 in CFA (Difco Laboratories, Detroit, MI). Booster immunizations consisted of a second intraperitoneal injection of 100 µg of HGG in sterile, pyrogen-free PBS.

Flow Cytometry

Approximately 5-6 weeks after bone marrow transfer 200 µl of tail vein blood was collected into heparinized tubes. PBMCs were isolated using Lympholyte M (Cerdarlane Laboratories, Hornby, Ontario, Canada) density gradients. The cells were then collected into PBS, supplemented with 3% FCS and 0.1% NaN_3. For cell surface staining, saturating amounts of biotinylated D3-137.5 (mouse IgG2a anti-I-A^b)(51) and fluoresceinated TIB 92 (mouse IgG2a anti-I-A^b)(67) or biotinylated AF6-78.25 (mouse

IgG1 anti-IgM^b)(68) and fluoresceinated DS-1 (mouse anti-IgM^a)(69) or biotinylated HIS51 (mouse IgG2a anti-CD90.1) and fluoresceinated 30-H12 (rat IgG2b anti-CD90.2) were used as the first step. The second step consisted of incubating with phycoerythrin (PE)-conjugated streptavidin (Jackson ImmunoResearch, West Grove, PA). The cells were then washed three times in PBS and fixed with an equal volume of 2% paraformaldehyde. List mode data was acquired on a FACScan flow cytometer (Becton Dickinson, Palo Alto, CA) using PC Lysis software. Dead cells were excluded by forward and side scatter gating. List mode files were then analyzed using Lysis II software. Monoclonal antibodies were labeled either with biotin hydrazide (53) or fluorescein isothiocyanate (FITC) (54), as needed. At the study termination the same procedures were used with the exception that splenocytes were used for cell staining.

Indirect Immunofluorescence

Sera from mice collected 5 weeks after the initiation of $HgCl_2$ was tested for the presence of ANoA by indirect immunofluorescence using commercially prepared mouse frozen kidney slides (Sanofi, Chaska, MN). The slides were incubated with sera diluted 1:50 in PBS for 30 minutes at room temperature. For nonallotype-specific ANoA the slides were incubated for 30 min at room temperature with FITC-conjugated goat anti-mouse IgG (Fc fragment specific, Jackson ImmunoResearch, West Grove, PA) diluted 1:50 in PBS. For the allotype-specific ANoA determination slides, were incubated with rabbit anti-mouse IgG2a^a or IgG2a ^{b} (Nordic Labs, Capistrano Beach, CA) followed by FITC-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA).

These reagents had been pre-titrated by ELISA against an allotype-nonspecific rabbit anti-IgG2a antibody (Nordic Labs) to produce equivalent sensitivities. Antinucleolar staining was evidenced by intense homogeneous staining of the nucleoli.

Allotype-Specific ELISA

The protocols used for measurement of allotype-specific serum total IgM and IgG2a were minor modifications of previously described procedures (63). For serum total IgM, samples were developed with affinity-purified donkey anti-mouse IgM (Jackson ImmunoResearch, West Grove, PA). IgM^a and IgM^b were measured using DS-1 (69) and AF6-78.25 (68) respectively. Serum total IgG2a was determined using rabbit anti-mouse IgG2a (Nordic Labs, Capistrano Beach, CA) while allotype-specific IgG2a was measured using either rabbit anti-mouse $IgG2a^a$ or $IgG2a^b$ (Nordic Labs, Capistrano Beach, CA). These reagents had been pre-titrated by ELISA against the allotype-nonspecific rabbit anti-IgG2a antibody (Nordic Labs) to produce equivalent sensitivities. The rabbit antibodies were detected using alkaline phosphatase-conjugated donkey anti-rabbit IgG (Jackson ImmunoResearch, West Grove, PA). Allotype-specific IgG2a anti-HGG was measured using the same protocol except that HGG was used as the first step.

Results

MHC-Restricted T Cell Help Was Required to Produce ANoAs

Exposure to $HgCl_2$ induces a wide range of physiologic effects in mice, including the release of large amounts of cytokines, especially the Th2 cytokine IL-4. It was

therefore possible that the antifibrillarin response could result from non-MHC-restricted interactions in genetically susceptible mice. To test this possibility, B6.TC mice were reconstituted with T cell-depleted B6.SJL marrow. Because intrathymic positive selection of T cells is mediated by the radioresistant thymic cortical epithelial cells (70), in these mice all CD4+ T cells would be positively selected to interact with the host I-A^b and not the donor I-A^s haplotype. Therefore, the B6.SJL-derived B cells would not be expected to receive MHC-restricted help. In contrast, central tolerance is mediated by bone marrow-derived dendritic cells although thymic epithelial cells can make a significant contribution (71), and therefore the animals would be tolerant to both haplotypes. Flow cytometric analysis of peripheral blood lymphocytes showed that all of the B cells were of donor B6.SJL origin. Surprisingly, despite the presence of these susceptible B cells and the massive activation of cytokines following $HgCl_2$ administration none of the animals developed ANoAs (Table 3-1). The addition of B6.TC cells in the inoculum did not alter the outcome. In marked contrast, syngeneically reconstituted B6.SJL mice responded well to mercury chloride, demonstrating that lack of responsiveness was not an artifact of radiation. We therefore conclude that the antifibrillarin specificity is mediated by I-A^s - restricted T cell help.

Table 3-1 MHC-restricted T cell help was required to produce ANoAs[a]

Donor	Host	n[b]	ANoA Positive (%)[c]
B6.SJL & B6.TC	B6.TC	4	0
B6.SJL & B6.TC	B6.SJL	5	100
B6.SJL	B6.SJL	7	100
B6.SJL	B6.TC	5	0

[a]ANoA, antinucleolar antibodies
[b]Number of mice
[c]Fraction of positive mice

Absence of ANoA Production in Haplotype-Heterozygous Mice Was Not Due to a Difference in Thymic Education

Although the above experiments showed the importance of T-B cell collaboration it is possible that I-A^b expression in the host and/or donor affected thymic education and therefore eliminated a population of responsive T cells. This led us to investigate the responses in (B6.SJL x B6.TC)F_1 hosts. When (B6.SJL x B6.TC)F_1 mice were used as hosts and reconstituted with B6.SJL, they all (12/12) responded to $HgCl_2$. When the same hosts were used but the donor changed to B6.TC, none (0/6) of the mice produced ANoAs (Table 3-2). Therefore, the donor haplotype determined whether or not autoantibodies are produced when developing cells were positively selected on both haplotypes.

Allogeneic Mixed Chimeras Developed ANoA Titers in Response to $HgCl_2$

In the rat model of mercury chloride-induced autoimmunity, allogeneic mixed microchimerism has been able to induce a state of tolerance (72). In contrast, other models have shown the potential for epitope spreading once tolerance is broken (73,74).

Therefore, it was of interest to determine whether the co-existence of B cells sensitive and resistant to the effects of $HgCl_2$ could influence each other.

To test these possibilities, we lethally irradiated (B6.SJL x B6.TC)F_1 mice and transferred T cell-depleted bone marrow from the following: 1) B6.SJL alone; 2) B6.TC alone; 3) a combination of B6.TC & B6.SJL; and 4) (B6.SJL x B6.TC)F_1 alone. In all four groups, T cells would be positively selected by either I-A^s or I-A^b. After confirmation of mixed chimerism in group 3 (see below), the mice were treated with $HgCl_2$ and the results are shown in Table 3-2. As expected, (B6.SJL x B6.TC)F_1 mice reconstituted with only B6.SJL bone marrow responded readily to $HgCl_2$ while mice given only resistant B6.TC bone marrow failed to develop an ANoA antibody titer. In contrast, syngeneic reconstitution with resistant (B6.SJL x B6.TC)F_1 bone marrow resulted in a very poor ANoA response. These results essentially duplicated our experience in analogous non-chimeric $HgCl_2$-treated B6.SJL, B6.TC, and (B6.SJL x B6.TC)F_1 mice (8) and again demonstrated that radiation did not affect this outcome.

Strikingly, mice given a combination of resistant and susceptible bone marrow had an intermediate result, with nearly 50% of the mice responding (Table 3-2). By allotype-specific ANoA, all of the responders produced exclusively b allotype autoantibody and therefore of donor B6.SJL origin (data not shown). This is not due to an intrinsic property of the a allotype, for the a allotype B6.SJL-Igh^a strain responded equally well to mercury (data not shown). Thus, the presence of ANoA in these mice resulted from the loss of tolerance to fibrillarin in I-A^s B cells.

Table 3-2 ANoA[a] response in chimeric (B6.TC x B6.SJL)F_1 host mice

Group	Source of Donor Bone Marrow	n[b]	ANoA Positive (%)[c]
1	B6.SJL	12	100
2	B6.TC	6	0
3	B6.SJL & B6.TC	16	43.8
4	(B6.SJL x B6.TC)F_1	17	11.8

[a]ANoA, antinucleolar antibodies
[b]Number of mice
[c]Fraction of positive mice

Lack of ANoA in Non-Responsive Mixed Chimeras Was Unrelated to B Cell Composition

Of interest, the response rate of 44% in the mixed chimeras (Group 3) was significantly different than either the 100% seen in Group 1 ($p<0.02$ by z-test for proportions) or the 11% seen in Group 4 ($p<0.04$) and suggested that the presence of resistant class II could exert a negative influence even when not co-expressed on the cells otherwise capable of responding. To evaluate for the possibility that this effect was merely due to dilution of potentially susceptible I-A^s-bearing B cells by resistant I-A^b B cells, we compared the B cell makeup of the positive and negative mice. The results are shown in Figure 3-1. The median % of I-A^s expressing B cells was 58.5% in those mice which failed to produce ANoAs while it was 60 % in those mice that did (Figure 3-1A). Even mice which had as few as 38% of their B cells from the sensitive parent were able to produce autoantibodies. Comparable results were seen when analyzed by B cell allotype (Figure 3-1B). Therefore, these data suggest that the decreased responsiveness in the presence of resistant B cells was not just a dilutional effect.

B Cells of Both B6.TC and B6.SJL Origin Were Functional in Group 3 Mixed Chimeras

It was possible that the presence of a and b allotype B cells in the mixed chimeras influenced the development of antinucleolar antibodies through antigen non-specific mechanisms. For example, in some combinations, allotype-specific suppression of immune responses has been seen (75). This possibility was minimized by the use of allotype-heterozygous host mice. However, to assess further the functionality of B cells of both B6.SJL and B6.TC origin, allotype-specific total IgM and IgG2a ELISAs were performed. In addition, the mice were also assayed for their response to immunization with a T cell dependent antigen, HGG. As shown in Figure 3-2, total IgM and IgG2a of both allotypes were present. Interestingly, despite the predominance of IgM of B6.SJL origin, IgG2a was much better balanced between the two donors. Moreover, there was a good IgG2a response to a T cell dependent antigen by B cells of both B6.SJL and B6.TC origin (Figure 3-3A), and both ANoA positive and negative mice responded equally well (data not shown).

Lack of $HgCl_2$-Induced ANoA Response by Haplotype-Heterozygous Mice Was Not Due to Absence of Anti-Fibrillarin-Specific I-A^s-Restricted T Cell Help

To determine whether or not the intermediate response seen in the mixed chimeras and in $HgCl_2$-resistant (B6.SJL x B6.TC)F_1 mice was due to a lack of I-A^s-restricted T cell help, we reconstituted (B6.SJL x B6.TC)F_1 mice with a combination of syngeneic (B6.SJL x B6.TC)F_1 and B6.SJL bone marrow. Fortyfive percent (5/11) of these mice produced

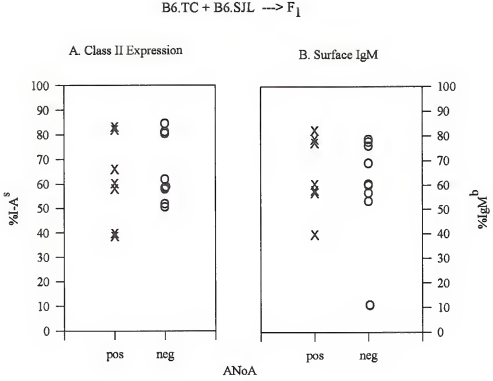

Figure 3-1 Immunofluorescence on peripheral blood lymphocytes of parental into F_1 chimeras. Data are presented as percent of cells expressing class II. A, Class II expression; B, surface IgM.

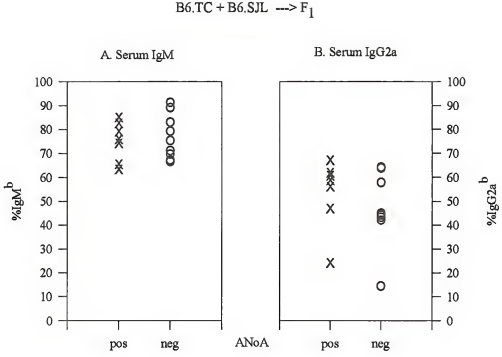

Figure 3-2 Allotype-specific total IgM and IgG2a ELISA data of parental into F_1 chimeras. Data are presented as a percentage of total isotype. A, Serum IgM; B, serum IgG2a.

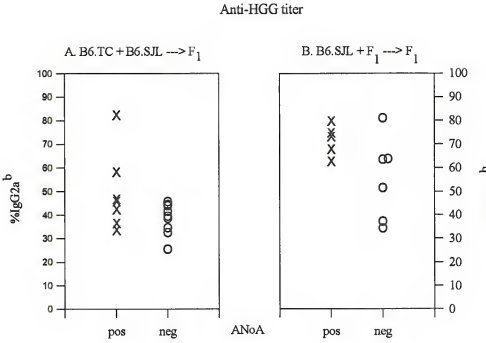

Figure 3-3 Allotype-specific anti-HGG ELISA data. Data are presented as a percentage of total isotype. A, B6.TC + B6.SJL ---> F_1 mice; B, B6.SJL + F_1 ---> F_1 mice.

ANoAs upon $HgCl_2$ treatment. The fact that some of the mice responded meant that T cells were available in which tolerance to fibrillarin was broken. Allotypic analysis revealed that all of the autoantibodies were of the b allotype. It seems likely that they arose from the B6.SJL donor since the B cells from the (B6.SJL x B6.TC)F_1 mice would have produced autoantibodies of either a or b allotype and no a allotype autoantibodies were detected even at a 1:10 dilution (data not shown). Thus, despite the presence of T cells which provided help in an I-A^a-restricted fashion, those B cells which co-expressed both haplotypes did not produce autoantibodies.

B Cell Composition Was Not a Determining Factor in Responsiveness

Figure 3-4 shows the B cell makeup of the B6.SJL + (B6.SJL x B6.TC)F_1 ---> (B6.SJL x B6.TC)F_1 mice. Flow cytometric analysis of class II expression showed these mice to be very well balanced (%I-$A^{a/b}$ range: 33-54%). The predominance of IgM of the b allotype (61-82%) is expected considering that approximately one-half of the B cells from the (B6.SJL x B6.TC)F_1 donor would express IgM of the b allotype. Similar to the parental into (B6.SJL x B6.TC)F_1 mice no correlation was noted between the B cell makeup and responsiveness (Figure 3-4).

Both Bonors Provided Functional B Cells

The apparent lack of response from the a allotype B cells led us to examine whether these B cells were functionally equivalent. Figure 3-5 shows that IgM and IgG2a of both allotypes were produced although the composition is somewhat skewed to the b allotype. Again, this is an expected finding since the cells from the (B6.SJL x B6.TC)F_1

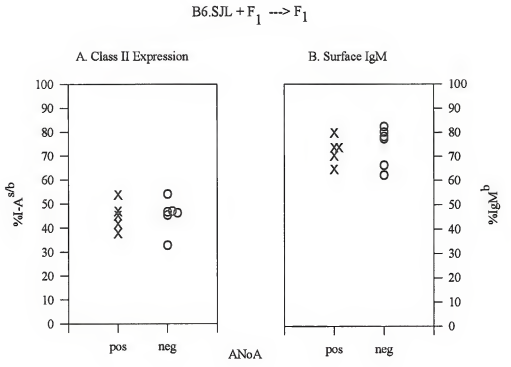

Figure 3-4 Immunofluorescence on peripheral blood lymphocytes of B6.SJL + F_1 ---> F_1 chimeras. Data are presented as percent of cells expressing class II. A, Class II expression; B, surface IgM.

mice would produce either a or b allotype. Similarly, B cells of the a allotype responded equally well when the response to a T cell dependent antigen (HGG) was analyzed in an allotypic fashion (Figure 3-3B). A comparison between positive and negative mice revealed no significant differences. Taken together, we conclude that the inability of haplotype-heterozygous mice to respond to $HgCl_2$ with an ANoA response is an intrinsic property of the B cells.

Absence of ANoA Production in Haplotype-Heterozygous Mice Was Not Due to the Presence of I-A^b-Restricted T Cells

It was possible that the non-responsiveness of the mixed chimeric as well as the syngeneically reconstituted (B6.SJL x B6.TC)F_1 mice was due to a negative regulatory effect of I-A^b -restricted T cells. To test for this possibility we lethally irradiated B6.SJL mice and transferred T-cell-depleted bone marrow from (B6.SJL x B6.TC)F_1 mice. To ensure no carry over of T cells educated in an (B6.SJL x B6.TC)F_1 mouse we treated the recipients at the time of transfer with 0.1 mg i.p. of MmT1 (mouse IgG2a anti-CD90.2 (66)). The T cells in these mice are unable to provide help through I-A^b since they developed in an I-A^s expressing host. Flow cytometric analysis confirmed that all the B cells in these mice expressed both haplotypes. Interestingly, none (0/11) of the mice produced ANoAs following $HgCl_2$ treatment. The resistance of these mice could not have been due to negative influences by I-A^b -restricted T cells.

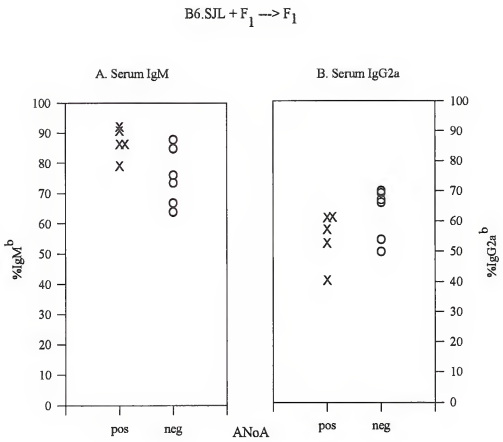

Figure 3-5 Allotype-specific total IgM and IgG2a ELISA data of B6.SJL + F_1 ---> F_1 chimeras. Data are presented as a percentage of total isotype. A, Serum IgM; B, serum IgG2a.

Discussion

$HgCl_2$ treatment in mice induces a wide range of physiological responses including marked increases in IL-4 production (20,25). The requirement for T cells in this model was shown by the fact that nude mice on the H-2^s background fail to develop autoantibodies (15). Splenic CD4+ T cells of $HgCl_2$-treated H-2^s mice have been shown to have a strong increase in IL-4 mRNA, whereas those of H-2^d mice showed only a weak increase (17). Interestingly, treatment of H-2^s mice with anti-IL-4 monoclonal antibody did not prevent ANoA production although it changed the isotype profile of the autoantibodies (25). Despite the possibility for non-cognate help via cytokines, we have shown that MHC-restricted T cell help was required to produce ANoAs. The B cells were not merely responding to cytokines in the environment but required specific MHC-restricted signals from T cells.

Cognate interactions are also required in several other models of autoimmune disease. Double parental-into-F_1 chimeras were used in the graft-vs-host model to show that autoantibodies were derived nearly entirely from B cells receiving direct alloreactive T cell help (51). Bone-marrow chimeras were also used to show that autoantibody production in *lpr* mice was restricted to those B cells that received T cell help (76). Thus, in three very different murine models of autoimmunity, in vivo autoantibody responses were MHC-restricted.

When the difference in responsiveness between B6.SJL ---> B6.TC and B6.SJL ---> B6.SJL mice was examined it became apparent that the possibility existed for significant differences in the T cell repertoire. Using bone marrow transfers involving

class II deficient mice Glimcher *et al* (77) showed that it is the radioresistant host thymic epithelial cells which mediate positive selection. In our model it was possible that lack of expression of I-A^s on the radioresistant thymic population eliminated responsive T cells. The use of (B6.SJL x B6.TC)F_1 mice as hosts allowed for positive selection on both I-A^s and I-A^b and led us to conclude that responsiveness is dependent on the donor haplotype when T cells for both haplotypes are positively selected.

Mixed chimerism has been shown to prevent autoimmunity in several systems. Nonobese diabetic mice were protected from diabetes when they were made chimeric with diabetes resistant bone marrow (78,79). It appeared that the autoimmune potential of the NOD cells was restrained. However, when the amount of resistant cells was decreased, a low incidence of insulinitis was seen. Using the rat model of mercury-induced autoimmunity Delaney *et al.* (72) showed that the presence of resistant bone marrow cells (microchimerism) converted an otherwise sensitive rat strain to a resistant one. If similar to the rat model, it would be expected that the presence of immune competent cells of resistant origin would prevent B6.SJL-derived B cells from responding to $HgCl_2$ with an antifibrillarin response. Our experiments differed somewhat in that we used a resistant (B6.SJL x B6.TC)F_1 mouse which had been coinfused with a combination of resistant and sensitive bone marrow. However, B6.SJL alone as the bone marrow donor completely restored the ability to produce ANoAs upon treatment with $HgCl_2$. Despite containing a significant amount of resistant cells we still obtained an intermediate response.

The presence of autoantibodies could play a role in spreading autoimmunity from a dominant epitope to previously cryptic epitopes (80). Presumably, binding of antibody can alter antigen processing, revealing new epitopes. Several other investigators have

shown that is it possible to break T cell tolerance to self antigens by coimmunizing mice with self and foreign antigens which in turn generates cross-reactive B cells that can elicit an autoimmune T cell response to previously cryptic self determinants on the autoantigen (73,74,81,82). These forms of epitope spreading do not appear to be occurring in our model. The production of autoantibodies by the B6.SJL B cells in the B6.SJL + (B6.SJL x B6.TC)F_1 ---> (B6.SJL x B6.TC)F_1 and the B6.SJL + B6.TC ---> (B6.SJL x B6.TC)F_1 mice did not result in the loss of tolerance to fibrillarin by previously resistant B cells.

Several other differences exists between our model and that used by Delaney (72). To induce chimerism without myeloablation the rats were transiently treated with the immunosuppressive agent FK506. Some protection from the manifestations of $HgCl_2$-induced autoimmune disease was seen in the rats receiving FK506 alone. The authors acknowledged that the results do suggest that transient immunosuppression was an important component of the protection. We used a complete myeloablation procedure which allowed us to forego any immunosuppressive treatment. Another important difference lies in the fact that in the rat model regulatory T cells appear which render the rats resistant to additional $HgCl_2$ injections and can confer resistance to naive rats (83). Regulatory T cells have not been identified in the mouse model and the animals do not become resistant to further treatment.

The intermediate response seen in group 3 (Table 3-2) mice was a surprising finding. It was possible that the ANoA negative mice failed to respond because the number of potentially responsive B cells of I-A^s origin had been reduced by the presence of resistant B cells of I-A^b origin. This mechanism appears unlikely in as much as the B

cell composition of the chimeras had no relationship to the development of an ANoA response. Another antigen non-specific mechanism which could have accounted for the lack of response involves allotype suppression. For example, in studying chronic graft-vs-host disease Morris *et al.* found that in allotype-heterozygous recipients, the autoantibodies were preferentially made by those host cells that expressed the donor allotype, whereas those host B cells that expressed nondonor allotype were relatively suppressed. In allotype-homozygous recipients, the donor cells frequently suppressed the host allotype completely (75). To minimize the possibility that this phenomenon could be occurring in our mice we used allotype-heterozygous mice as hosts. Allotypic analysis of spontaneous as well as antigen specific antibodies demonstrated good participation by both allotypes. Therefore, allotype suppression was not a factor in our mixed chimeras.

A more likely possibility for the poor responsiveness of haplotype-heterozyous mice was lack of specific T cell help. By substituting (B6.SJL x B6.TC)F_1 for B6.TC as the resistant donor in mixed chimeras also receiving susceptible B6.SJL bone marrow, we provided a mechanism to verify the presence of I-A^s-restricted T cell help in individual chimeric mice. In those mice with a positive ANoA, T cells capable of helping antifibrillarin-expressing B cells of I-A^s origin must be present, and these activated T cells, particularly with their reduced stimulation threshold, should also be capable of interacting with antifibrillarin-expressing B cells of I-$A^{s/b}$ origin. Moreover, by using haplotype-heterozygous mice as co-donors, the overall expression of the resistant I-A^b haplotype was reduced in cells of donor origin. Despite this, there was no increase in ANoA response rate. More surprisingly, in the ANoA positive chimeras, this specificity was limited to the

b allotype, indicating that the haplotype-heterozygous B cells failed to participate in this response. These results strongly suggest that lack of responsiveness is an intrinsic regulatory property of B cells.

The presence of I-A^b restricted T cell help could have accounted for the intermediate response seen in the mixed chimeras. The (B6.SJL x B6.TC)F_1 mice that served as hosts provided an environment in which T cells would be selected to interact with I-A^b expressed on the B cells from the B6.TC hosts. By transferring (B6.SJL x B6.TC)F_1 bone marrow into B6.SJL mice, there would be little potential for positive selection of T cells on I-A^b. Despite the absence of these cells, there was no ANoA response to $HgCl_2$. This again points to an intrinsic defect in (B6.SJL x B6.TC)F_1 B cells.

One possibility for this intrinsic property is the concept of MHC-guided processing leading to determinant capture (84). This hypothesis states that when an antigen is taken up by APCs and begins unfolding, different MHC molecules can compete for determinants. Once it is bound by class II, the antigen is then trimmed down to its final size while the remainder of the antigen including cryptic epitopes is discarded. An example of this phenomenon was seen in the autoimmune disease insulin-dependent diabetes mellitus (85). The response to the subdominant A^{NOD}-restricted determinant of HEL disappears when NOD mice were made transgenic by introduction of the E_α^d. The responsivness was restored when scission of the HEL separated this determinant from its adjoining, competitively dominant, E^d-restricted determinant. This suggested that the E^d molecule bound and protected its dominant determinant on a long peptide while captured neighboring determinants were lost during proteolysis. In our mice, I-A^b could effectively

be binding fibrillarin in the (B6.SJL x B6.TC)F_1 cells, thereby preventing I-A^s from presenting fibrillarin and receiving T cell help.

Recently the molecular and antigenic properties of mercury-modified fibrillarin has been examined. The exposure of fibrillarin both in vivo and in vitro caused a change in its migration under non-reducing SDS-PAGE and resulted in a loss of reactivity to autoantibodies. Mutation of the cysteines in fibrillarin resulted in a loss of mercury-induced modification. The authors concluded that unmodified fibrillarin is the B cell antigen while the T cell antigen appears to be mercury-modified fibrillarin (38). Therefore, if our model is correct, the presence of I-A^b affected the processing of Hg-modified fibrillarin in the context of I-A^s and provide a unique opportunity for testing the role of antigen competition in an important environmental model of induced autoimmunity.

CHAPTER 4
DISCUSSION/CONCLUSION

Humans are continually exposed to mercury via various routes resulting in high levels in skin, nails, hair and the kidneys. Many exposures are a result of mercury being widely distributed in the environment. The major natural source of mercury is the degassing of the earths crust, and is estimated to produce between 2700 to 6000 tons per year. Approximately 10,000 tons of mercury are mined each year. Even industrial activities not directly employing mercury or mercury products give rise to substantial amounts. Fossil fuels, for example, may contain up to 1 ppm resulting in 5000 tons per year being emitted from burning coal, natural gas and from refining petroleum products (86). Human are also exposed to mercury in the form of pharmaceuticals, cosmetics and dental amalgams.

On the basis of toxicologic characteristics, there are three forms of mercury; elemental, inorganic, and organic compounds. Both organic and inorganic forms of mercury undergo environmental transformation. Metallic mercury may be oxidized to inorganic divalent mercury, particularly in the presence of organic material. Divalent mercury may, in turn, be reduced to metallic mercury when reducing conditions are present. Anaerobic bacteria are capable of methylating the divalent form resulting in dimethyl mercury. In tissues, methyl mercury undergoes biotransformation to divalent mercury compounds (86).

Metallic or elemental mercury volatilizes to mercury vapor at ambient temperatures, and most human exposure is by inhalation. Acute exposure can result in corrosive bronchitis and interstitial pneumonia. Long term exposure to mercury vapor targets the central nervous system producing a triad of signs including; increased excitability, tremors and gingivitis. Renal effects are sometimes seen with chronic mercury vapor exposure and may be similar to that which occurs following inorganic mercury exposure. The brain is also a target organ for methyl mercury. Environmental exposures, such as eating mercury containing fish can result in neurotoxic effects. These include parathesia, ataxia, neurasthenia, vision and/or hearing loss, tremors and possibly coma followed by death (86).

Inorganic mercury salts may be present as divalent (mercuric) or monovalent (mercurous). Ingestion of high doses of this form of mercury causes corrosive ulceration and necrosis of the gastrointestinal tract followed by circulatory collapse. If this initial insult is survived, renal failure occurs within 24 hours due to necrosis of the proximal tubular epithelium.

Chronic low-dose exposure to inorganic mercury can cause immunological aberrations in both humans and rabbits (27). Immunosuppressive effects have been reported in mice resulting in impaired host resistance (87). The immunoactivating properties of inorganic mercury in mice have been widely studied and can be divided into three major pathological sequelae: lymphoproliferation, hypergammaglobulinemia and the development of autoimmunity (27). Lymphoproliferation is classified as an MHC-independent effect while autoimmunity development is tightly restricted by haplotype.

Currently, the link between MHC haplotype and susceptibility to $HgCl_2$ induced ANoAs has remained unexplained. The experiments conducted for this dissertation were begun with the idea of examining just how the MHC haplotype determines the response to $HgCl_2$. Through the use of H-2-recombinants it has been shown that responsiveness could be mapped to the I-A region of the MHC (6). Mice of the H-2^s haplotype are high responders while those of the H-2^b haplotype are resistant (5). In an F_1 cross between two different haplotypes, the products of both alleles are expressed on the same cell, so expression is said to be co-dominant. Therefore, an F_1 (s/b) mouse should be susceptible to ANoA induction. This result has been reported in the literature although the response was somewhat attenuated (5,9,43). When we generated s/b haplotype mice using H-2-congenic founder strains, we surprisingly found that the mice were resistant. This led us to examine how the resistant haplotype was exerting its effects.

It was possible that a gene linked to the resistant haplotype and not the I-A molecule itself that could be preventing the response. To this end we generated F_1 mice between resistant and susceptible strains in which the resistant class II genes were eliminated. These mice did develop ANoAs which showed that the downregulation was a result of the resistant allele itself and not a linked gene.

We also directly examined what effects, if any, that the other class II molecule, I-E, had on susceptibility. Previous studies in the murine mercury model have shown expression to either suppress (6) or have no effect (88) on susceptibility. The protection afforded by I-E is not without precedent. Expression of I-E in lupus prone B6.lpr mice significantly reduced the production of autoantibodies as well as lowering the spleen and

lymph node weights (48). Using bone marrow chimeras in which I-E-positive and I-E-negative B cells co-existed, it was determined that the amelioration of signs resulted from a direct effect of I-E on the B cell This phenomenon may be a universal feature of autoimmune disease on the H-2B background. In an unrelated autoimmune mouse model, BXSB, which also bears the $H2^b$ haplotype, expression of I-E prevented the development of SLE (47), and a potential mechanism was proposed. It was shown that the α chain of the I-E molecule generates a peptide with high affinity to $I\text{-}A^b$, thereby competing with the pathogenic autoantigen-derived peptides for presentation to B cells. Interestingly, if this same mechanism were important in the $HgCl_2$ model, we would predict just the opposite results, in our (B6.SJL X B6.I-E)F_1 mice. Since the that lack of response in s/b mice was due to expression of $I\text{-}A^b$, the I-E α-derived peptide could have preferentially blocked $I\text{-}A^b$ and enhanced the $I\text{-}A^s$-mediated ANoA response. However, we found no enhancing effect on autoimmunity by co-expression of I-E on F_1 mice. One possibility for this lack of effect was that an I-E-derived peptide might also have an equal or even greater affinity for $I\text{-}A^s$. Against this possibility was our results that co-expression of I-E on the $H\text{-}2^s$ homozygous background did not attenuate the autoimmune response, as would be expected based on the experience with the BXSB and lpr models of autoimmunity. Therefore, the lack of autoantibody production in these F_1 mice led us to investigate other mechanisms whereby $I\text{-}A^b$ could be down regulating the response.

For these studies, we turned to bone marrow chimeras. Adoptive bone marrow transfer is an important tool that can be utlitized to dissect the cellular interactions in an adaptive immune response by manipulating the environment in which T cell development occurs thereby modifying the T cell repertoire. By taking advantage of the differences in

positive and negative selection seen between host and donor cells we showed that MHC restricted T cell help was required to produce ANoAs. This result was not surprising but was instrumental in interpreting future results. The next possibility we examined was whether or not the differences in T cell repertoire could account for the differences in susceptibility. By using an F_1 cross between the two donor strains we eliminated the T cell differences yet still saw differences in responsiveness.

The intermediate response seen when resistant and sensitive bone marrow was coinfused into a haplotype heterozygous host was surprising. Several possibilities could have accounted for this. The first possibility, dilution of responsive B cells, was ruled out by noting a lack of any correlation between B cell composition and responsiveness. Secondly, we needed to rule out that allotype suppression in which autoantibodies are preferentially produced by those B cells expressing the same haplotype as the host was occurring in these chimeras. We attempted to eliminate this possibility by using F_1 hosts. We also verified that allotype suppression was not an important mechanism in the chimeras by observing a brisk response by both allotypes when immunized with an exogenous antigen. The final possibility we examined that may have led to the intermediate result involved the question of T cell help. By using F_1 rather than B6 mice as the resistant co-donor and observing an anti-nucleolar response by the B cells of donor B6.SJL origin, we were assured that activated T cells capable of providing I-A^s-restricted help to antifibrillarin B cells were present. Despite the presence of adequate T cell help that should have been able to interact with (s/b)F_1 B cells, no increase in susceptibility was observed. The fact that these mice produced ANoA only of the b allotype combined with

the observation that no response was seen when I-A^b restricted T cells were eliminated led us to conclude that the resistance of the F_1 B cells was due to an intrinsic defect of the B cells themselves.

Several theories have been proposed as to how mercury can lead to autoimmunity. It has been suggested that mercury may alter self antigen by complexing with the antigen itself. As mentioned above, divalent mercury is a highly reactive molecule with a propensity to bind sulfhydryl groups of proteins and nonprotein thiols. Reactivity has also been found towards hydroxyl, carboxyl and phosphoryl groups albeit at a lower affinity (89). These modified self antigens may then be recognized by the immune system as foreign and in turn induce an immune response capable of recognizing the modified self antigen.

During T cell development, self tolerance is only induced to efficiently-presented dominant epitopes but not to cryptic ones (90). Therefore, potentially autoreactive T cells which recognize these cryptic epitopes that would arise by random rearrangement of the T cell receptor would not be eliminated by negative selection. The dominant epitope presented is influenced by a number of factors and is incompletely understood. Factors that have been described include the haplotype, protein structure and/or conformation. Changes in any of these factors could theoretically result in novel cleavage products. This phenomenon has been observed for many of the autoantigens recognized by SLE patients. Casciola-Rosen *et al.* (91) showed that during apoptosis the lupus autoantigens cluster and are concentrated in the surface blebs of apoptotic cells. This could result in the autoantigens becoming substrates for enzymes not present within the cell resulting in new

cleavage products. A follow-up study by the same group (90) showed that several of the autoantigens targeted in scleroderma are susceptible to reactive oxygen species in a metal-dependent manner. This cleavage generates unique epitopes resulting in autoantibody formation. They propose that the repetitive episodes of ischemia-reperfusion, which occurs in these patients as a result of vasomotor instability of their arterioles, are responsible for the generation of the reactive oxygen species. In contrast, fibrillarin was not fragmented under identical conditions, thereby suggesting an alternative mechanism was responsible for development of this specificity in a subset of scleroderma patients.

Pollard *et al.* (38) proposed a mechanism whereby mercury can induce antifibrillarin autoantibodies. They showed that mercury-induced cell death was associated with a loss of fibrillarin antigenicity and modification of the molecular properties of fibrillarin. By mutating the cysteines in fibrillarin to alanines they confirmed that $HgCl_2$ exposure *in vitro* leads to a disulfide bonded form of fibrillarin which is poorly recognized by autoantibodies using either indirect immunofluorescence or immunoprecipitation. This suggests that the mercury-induced change involves a conformational structure change that is no longer detected by autoantibodies.

The above mechanisms do not take into account the tight link between susceptibility to autoimmunity and the mouse MHC. We propose a model which elaborates on both the modified self-antigen theory and takes into account the lack of response seen in haplotype-heterozygous mice.

During T cell development, fibrillarin is processed and the immunodominant peptide(s) is presented. T cells which recognize this peptide are deleted resulting in

tolerance to fibrillarin. On exposure to $HgCl_2$, a disulfide bond forms which affects the immunodominant peptide normally presented by I-A^s, resulting in the presentation of a cryptic epitope. This cryptic peptide could potentially take a number of forms. The production of a mercury-modified disulfide link could protect the area around that peptide from being digested and the resultant cryptic peptide would be a normal sequence derived from another portion of the fibrillarin molecule. This would be a remote effect. It could also result from a conformational change that exposes a buried sequence to digestion. Mercury-modified disulfide could provide a completely novel peptide sequence centered around the cysteine-Hg-cysteine sequence. Arguing against this possibility is the fact that no mercury was detected in the glomerular basement membrane in $HgCl_2$-treated rabbits using autoradiography (92). Another possibility is that the disulfide residue could be re-reduced during processing but one of the amino acids in the binding groove has Hg-linked side chain that alters the immunogeneicity. The net result, then, would be the expression of a self-epitope not previously encountered. The immune system would recognize this as foreign, resulting in autoimmunity. In the case of a b haplotype mouse, the disulfide bond doesn't interfere with the processing and presentation of its immunodominant peptide. No cryptic epitopes are revealed and hence no autoimmunity to fibrillarin develops.

Our experiments with haplotype heterozygous mice adds some interesting potential insights into antigen processing and the expression of cryptic epitopes. We propose that the cryptic epitope presented by I-A^s is either the same or overlaps with the peptide normally presented by I-A^b. When an s/b haplotype mouse is exposed to mercury, a competition for access to that part of fibrillarin that could be bound by either of the two

class II alleles will result. If the I-A^b molecule has a greater avidity for this peptide than I-A^s, no new peptide is seen in the context of I-A^s and no autoimmunity results.

There is a precedent for the idea that different class II molecules actually compete for the same or overlapping peptide. Sercarz *et al.* (84) showed that competition takes place between I-A and I-E in the mouse. They coined the phrase "MHC guided processing" to describe the process where the MHC class II molecule would encounter a tightly folded protein antigen in one of the acidic compartments of the endosomal-lysosomal system soon after preliminary processing had succeeded in partially unfolding it, making one or a few determinants available. The dominant determinant would be the one that initially won the competition for binding to the MHC and provided the binding was stable, the residues bound within the MHC would be protected from further proteolysis or binding by a different MHC molecule. Thus far, this has been described in *in vitro* models. Our experiments in the mercury chloride-induced model would be the first demonstration of its potential importance *in vivo*.

Our investigations of mercury-induced autoimmunity in mice have stimulated the need for future investigations addressing the many questions spawned by our initial research into this area. Several major questions need to be addressed to either support or refute our model. Firstly, if in fact a competition is ongoing between MHC molecules in haplotype heterozygous mice, will increasing the dose of mercury which in turn should provide a greater pool of modified fibrillarin result in these animals now becoming susceptible? This is a fairly straightforward question to address. The second and perhaps more fundamental question to be addressed is the relationship between the T cell and B

cell epitopes. As previously discussed, the T cell epitope has been proposed to be mercury-modified fibrillarin, while the B cell epitope is a conformational determinant lost by exposure to mercury (38). This absence of an antibody response to mercury-modified fibrillarin is a surprising one. One possible explanation is that binding of the mercury-modified epitope by surface Ig protects that portion of the molecule from antigen processing. The net result is that no mercury-induced cryptic determinants are presented by those B cells with specificity for the mercury modification. While reasonable, this raises yet another issue: how then is it possible for a specific immune response to develop if the B cells that can bind to native fibrillarin do not have a mercury-modified T-cell epitope to present? One possibility is that the mercury either directly or indirectly causes an aggregation of fibrillarin in which some molecules are mercury-modified and some are still in the native conformation. When a B cell internalizes this aggregate specifically via its surface Ig receptor, which recognizes native fribrillarin, it processes the mercury modified sites within the aggregate and presents it to T cells, which recognize this modified form as foreign. While this hypothesis readily explains the potential for T-B interactions in the generation of a Hg-induced response to native fibrillarin it re-opens the question as to why there is no response to Hg-modified fibrillarin. It would seem unlikely that surface Ig could bind every Hg-modified residue in an aggregate. Failure to do so would allow the B cell specific for Hg-modified fibrillarin to present the cryptic epitope and thereby receive T cell help .

We have shown that it is the I-A^b molecule that confers resistance to $HgCl_2$-induced autoimmunity in F_1 (s/b) mice. Through the use of bone marrow chimeric animals

we determined that this effect is not mediated by a change in the T-cell repertoire. It appears that the MHC may be exerting its effects by manipulating the antigen processing/presentation pathway. To address this, one would need to elute and sequence the peptides that are actually being presented on the surface of the antigen presentation cells. Similarly, to address the apparent paradox between the T cell and B cell epitopes one would need to obtain the same information for both the T cell and the B cell receptors.

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BIOGRAPHICAL SKETCH

Gregory Alan Hanley was born in Batavia, New York, November 24, 1965. He received a Bachelor of Science degree from Geneseo State, a branch of the State University of New York. He then attended the University of Florida, College of Veterinary Medicine and was awarded the Doctor of Veterinary Medicine in 1993. After graduation Dr. Hanley received an NIH postdoctoral fellowship to pursue advanced training in the veterinary speciality of laboratory animal medicine and training in a basic science leading to a Ph.D. Dr. Hanley's focus during his studies concentrated on the induction of autoimmunity in mice using mercuric chloride, the results of which are included in this dissertation.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

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